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HIV-1 Vpu represents a minor target for cytotoxic T lymphocytes in HIV-1-infection

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We have previously shown that Vpu is rarely targeted by HIV-1-specific cytotoxic T lymphocytes (CTL). The present report extends these findings and describes the characterization of the first CTL epitope within HIV-1 Vpu, identified in an individual with long-term non-progressive HIV-1 infection. The epitope was shown to be highly conserved among HIV clade B sequences and is restricted by HLA-A*3303, an HLA allele commonly seen in Asian and west-African populations.

With more than 57 million individuals worldwide infected with HIV-1 since the beginning of the epidemic [1], the development of an effective HIV vaccine either to protect against HIV-1 infection or to attenuate the course of disease is urgently needed. Increasing evidence indicates that HIV-1-specific CD8 cytotoxic T lymphocytes (CTL) and CD4 T helper cells play a critical role in the control of viral replication in HIV-1 infection [2,3]. Recent studies have shown that HIV-1-specific CTL responses are directed against a large variety of different epitopes located within structural as well as regulatory and accessory HIV-1 proteins [4,5]. However, no HIV-1-specific CTL epitopes have been defined to date within the accessory HIV-1 protein Vpu, an 81 amino acid membrane protein that facilitates budding of new virus particles and regulates the degradation of CD4 cells [5,6].

In a previous report investigating CTL responses against all accessory proteins [5] we found detectable CTL responses against HIV-1 Vpu in one out of 60 (1.6%) individuals screened. In an extension of that study, we have now characterized HIV-1 Vpu-specific CTL responses in a total of 85 HIV-1-infected individuals at different stages of HIV-1 infection, including 45 individuals with treated primary infection, two individuals with untreated acute HIV-1 infection, 18 individuals with treated chronic infection, and 20 individuals with untreated chronic infection (14 long-term non-progressors and six progressors). HIV-1 Vpu-specific CTL responses were assessed using 14 overlapping peptides (15–18mer peptides overlapping by 10 amino acids) spanning the entire HIV-1 clade B Vpu sequence in an IFN-γ Elispot assay on fresh and frozen peripheral blood mononuclear cells (PBMC) [4,5]. In addition, all 85 individuals were tested for CTL responses directed against HIV-1 Gag and Nef using the same methods. CD8 T-cell dependence of all responses to synthetic peptides was confirmed by CD4/CD8 T depletion studies using magnetic beads (MACS; Miltenyi Biotech, Germany), as described [4,5].

While all study subjects had detectable HIV-1-specific CTL responses (92% against HIV-1 Gag and 80% against HIV-1 Nef), only two out of the 85 screened subjects (2.4%) had detectable, but low-level CTL responses directed against HIV-1 Vpu. Both individuals, an HIV-1 long-term non-progressor (CMW) [5] and one individual with primary HIV-1 infection (AC-71), had well-controlled viremia in the absence of antiretroviral therapy. These data confirm our previous reports on the remarkably low frequency of HIV-1 Vpu-specific CTL responses in HIV-1 infection compared with other HIV-1 proteins [5].

CD8 T cells in subject CMW targeted the overlapping peptide Vpu-6 (IVFIEYRKILRQRKID) with a frequency of 170 spot-forming cells/10^6 PBMC. It was found to be a subdominant response in this individual and contributed 5% to the total CTL response against the whole expressed HIV genome (data not shown). In order to confirm and verify the low-level CTL response in CMW, we generated Vpu-6-specific cytotoxic CD8 T cells using the limiting dilution assay [7]. As no HIV-1-specific CTL epitopes have been defined within HIV-1 Vpu to date [6], we set out to characterize the optimal CTL epitope within the Vpu-6 peptide. The HLA class I restriction of the Vpu-6-specific response was determined using an autologous and partially matched antigen-presenting lymphoblastoid B cell line (L-BCL) in a ^{51}chromium release assay, as previously described [4]. The response directed against the Vpu-6 peptide was shown to be restricted by HLA-A*3303, an HLA class I molecule frequently encountered in west Africa and southeast Asia [8,9] (Fig. 1a). The optimal sequence of the CTL epitope contained within Vpu-6 was characterized using PBMC and serial dilutions of peptide truncations in an Elispot assay [10]. As demonstrated in Fig. 1b, the nonamer peptide EYRKILRQR (ER9, Vpu 29-37)

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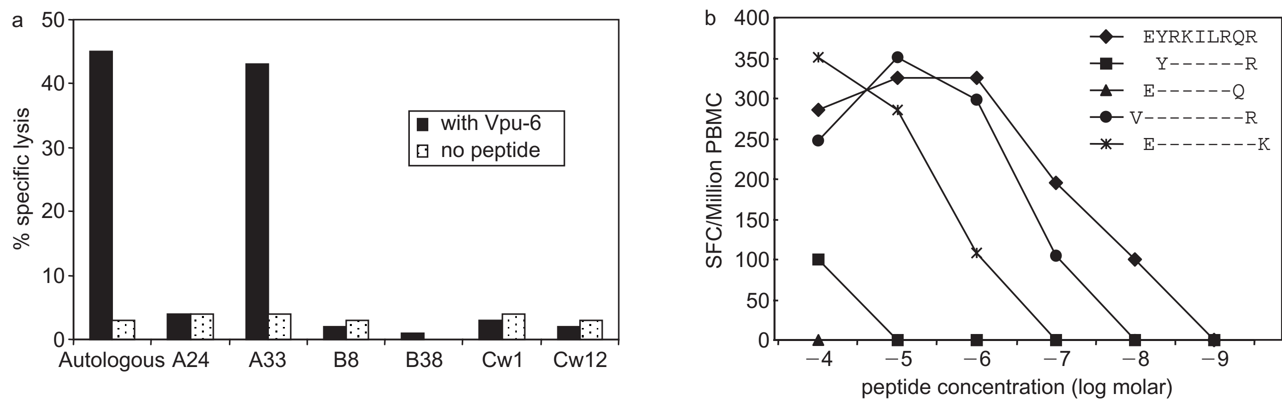

Fig. 1. (a) HLA restriction using autologous and partly HLA-matched lymphoblastoid B cell line (B-LCL) pulsed with the Vpu-6 peptide or no peptide as a negative control in a ^{51}chromium- release assay (E : T ratio 10 : 1). The HLA-A*3303 allele was identified as the restricting allele for the Vpu-6-specific cytotoxic T lymphocyte (CTL) response. (b) Fine-mapping of the optimal epitope contained within the Vpu-6 peptide using serial dilutions of peptide truncations in an Elispot assay, as previously described [4]. The peptide EYRKILRQR is identified as the optimal CTL epitope.

was recognized best at the lowest peptide concentration. The epitope lies within one of two α-helical regions in the cytoplasmatic domain of HIV-1 Vpu in proximity to the βTrCP binding motif, which may be implicated in the induction of apoptosis in HIV-infected cells [11]. Despite the high overall sequence variability observed for Vpu (Yusim *et al.*, personal communication), the epitope sequence is 71% conserved among the 60 published HIV-1 clade B Vpu sequences [12] (www.hiv.lanl.gov). The EYRKIL RQR epitope represents the first described optimal CTL epitope within the accessory protein Vpu, as well as one of the first HIV-1 CTL epitopes restricted by HLA-A*3303 [6,13]. Its sequence is in concordance with the described peptide-binding motif for HLA-A*3303 [14], containing a tyrosine at the P2 anchor position and an arginine at the C-terminal end of the peptide.

In order to assess the frequency of recognition of the newly defined epitope we also screened five additional HLA-A*3303-expressing HIV-1-infected individuals for responses to the EYRKILRQR epitope. Two of these five individuals and thus a total of three out of six (50%) tested HLA-A*3303-positive individuals had detectable CTL responses against the epitope, suggesting that it may in fact be a fairly common response among individuals expressing the HLA-A*3303 allele. Interestingly, all three HLA-A*3303-positive responders were HIV-1 long-term non-progressors with viral control in the absence of treatment.

These data confirm that HIV-1 Vpu is the least targeted protein by HIV-1-specific CTL, as measured using current techniques. This infrequent recognition by CTL is especially remarkable considering that another accessory protein of similar size Vpr (96aa) is highly targeted by CTL in HIV-1-infected individuals [5]. It has been demonstrated that HIV-Vpu is the most variable protein within HIV-1 (Yusim *et al.*, personal communication). Using overlapping peptides based on an HIV-1 clade B consensus sequence in the assays described here could therefore miss responses against this highly variable protein, if the autologous virus sequence is considerably different from the peptide sequence [15,16]. The characterization of HIV-1-specific CTL responses using peptides spanning the autologous virus sequence is needed to address the potential underestimation of immune responses directed against very variable HIV-1 proteins in the future. Further studies will thus be needed to assess whether this infrequent recognition of HIV-1 Vpu reflects low immunogenicity of the protein *in vivo*, a lack of HLA class I processing of Vpu, or an underestimation of CTL responses directed against this highly variable protein using peptides based on HIV-1 consensus sequences or other factors.

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CD4 cell count changes in individuals with counts above 500 cells/mm^3 and viral loads below 50 copies/ml on antiretroviral therapy

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We prospectively followed 166 individuals on antiretroviral therapy with viral loads less than 50 copies/ml and CD4 cell counts greater than 500 cells/mm^3 to ascertain CD4 cell count changes while the viral load remained below 50 copies/ ml. On average, CD4 cell counts remained stable and high. Only five patients experienced a decline to below 350 cells/mm^3, and these were typically isolated low values with CD4 cell percentages remaining high. Our findings suggest that it may be possible to reduce the frequency of CD4 cell count monitoring in individuals with a value greater than 500 cells/mm^3 so long as regular viral load monitoring indicates a value less than 50 copies/ml.

The CD4 cell count and viral load tend to be regularly monitored in individuals on antiretroviral therapy [1–5]. The current aim is to maintain viral load below 50 copies/ml, in order to minimize the risk of the development of resistance, whereas CD4 cell count monitoring is largely to assess the short-term risk of AIDS diseases [1,2,6]. However, increasing numbers of individuals on therapy have CD4 cell counts above 500 cells/mm^3, for which the associated risk of AIDS diseases is very low [6,7]. Furthermore, CD4 cell counts tend to increase or remain stable in individuals with viral loads less than 50 copies/ml on antiretroviral therapy [4,5,8]. This suggests that in individuals on antiretroviral therapy with viral loads less than 50 copies/ml and CD4 cell counts greater than 500 cells/ mm^3, it may not be necessary to monitor CD4 cell counts frequently, so long as the viral load remains less than 50 copies/ml. In order to investigate this further we assessed CD4 cell count changes after two consecutive values greater than 500 cells/mm^3 in individuals with viral loads continually less than 50 copies/ml on antiretroviral therapy.

We followed 166 patients from the Royal Free Clinic in London, from a time at which the viral load was less than 50 copies/ml and the CD4 cell count was greater than 500 cells/mm^3. Baseline (time zero) was the date of the second consecutive CD4 count value greater than 500 cells/mm^3. Follow-up was until the last viral load/ last CD4 cell count or the time of the first viral load greater than 50 copies/ml (whichever occurred first). All patients were on at least three antiretroviral drugs. CD4 cell counts were measured with a frequency of one per 10 weeks [interquartile range (IQR) 8–12 weeks], whereas the while viral load was measured with a frequency of one per 12 weeks (IQR 9–15 weeks).

The median CD4 cell count at time zero was 657 cells/mm^3 (IQR 582–790; range 501–1345), whereas the median previous CD4 cell count nadir was 253 (IQR 167–347; range 1–1294). At time zero patients had been on HAART for a median of 105 weeks. The

median follow-up time was 47 weeks (IQR 24–73), over which time a median (IQR) of four (two to six) viral load measures (all < 50 copies/ml, by design of the analysis) and four (three to seven) CD4 cell counts were performed. The median change in the CD4 cell count per 4 weeks, from time zero until the final value during follow-up, was +2 cells/mm^3 (IQR −9 to +12; range −286 to +127). The minimum value during follow-up ranged from 262 to 1222 cells/mm^3 (median 580; IQR 486–676). A total of 46 patients (28%) had at least one value less than 500 cells/mm^3. The median decline from time zero to this minimum value was 73 cells/mm^3 (range 0–500). There was only one occurrence of an AIDS disease during the total 162 person-years of follow-up; a lymphoma that occurred at a CD4 cell count of 635 cells/mm^3(CD4 cell count nadir 296 cells/mm^3).

We investigated further the five individuals (3%) who experienced a CD4 cell count below 350 cells/mm^{3}during follow-up. Details are given in Table 1. All five were aged under 50 years. The CD4 cell percentages at the time were available, and ranged from 22 to 32%. To put these values in perspective, we assessed the mean CD4 cell count according to the CD4 cell percentage, based on the first CD4 cell count in 2585 individuals in the entire Royal Free cohort. The median CD4 cell count among those with CD4 cell percentages of less than 5, 5–9, 10–14, 15–19, 20–24, 25–29, 30–34 and greater than 35% were 21, 129, 238, 344, 449, 548, 651 and 772 cells/mm^3, respectively. Table 1 also includes the subsequent value for the patients who experienced a CD4 cell count below 350 cells/mm^3. Each showed an increase.

Taken together, these results suggest that the CD4 cell count decreases to levels below 350 cells/mm^3 are rare in individuals with CD4 cell counts initially above 500 cells/mm^3 and with continuing viral suppression. When this does occur it seems likely to be transient and to be associated with a CD4 cell percentage that is higher than that normally observed in individuals with such a CD4 cell count. Factors such as rest before blood sampling [9] and early morning sampling [10] are known to be associated with lower CD4 cell counts and these could have played a role. It is also important to consider the possibility of falsely negative viral load values, for example caused by non-B subtype virus.

Table 1. Details of CD4 cell counts/percentagess experienced by five patients who experienced a CD4 cell count less than 350 cells/mm^3 despite an initial CD4 cell count greater than 500 cells/mm^{3}and a viral load continuously less than 50 copies/ml.

Baseline CD4 cell count (cells/mm^3)	Lowest CD4 cell count experienced (cells/mm^3) during follow-up	CD4 cell percentage	Next CD4 cell count after lowest (cells/mm^3)
532	262	22	374
740	330	30	705
650	331	32	792
560	347	27	392
642	349	28	404

The cost of diagnostic procedures such as CD4 cell counts are a small percentage of the total care costs [11], and newer technologies are resulting in considerably cheaper assays [12]. In addition, patients and clinicians use continuing high CD4 cell counts as positive feedback on the benefits of treatment. However, our results suggest that in those with a sustained viral load less than 50 copies/ml and two consecutive CD4 cell counts greater than 500 cells/mm^3, CD4 cell counts tend to remain stable and high. Further follow-up on extended numbers of patients in a variety of settings is required, but our findings suggest that it may be possible to reduce the frequency of CD4 cell count monitoring in those with a value greater than 500 cells/mm^3, so long as regular viral load monitoring indicates a value less than 50 copies/ml.

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Normalization of cytomegalovirus-specific CD4 T cells in HIV-1-infected individuals receiving antiretroviral therapy

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HIV infection is characterized by a loss of immunological function and the development of severe opportunistic infections such as cytomegalovirus. To determine the frequency of cytomegalovirus-specific CD4 T cells in healthy and HIV-infected individuals, we examined peripheral blood mononuclear cells from 10 HIV-negative and 55 HIV-positive individuals for antigen-induced intracellular cytokine responses. We found that HIV suppression with antiretroviral therapy leads to a normalization of previously increased cytomegalovirus-specific CD4 T cells, indicating the efficient control of an opportunistic pathogen.

Cytomegalovirus is one of the most frequent opportunistic pathogens in patients with AIDS [1]. Untreated HIV infection is characterized by a progressive loss of immunological function and the development of severe opportunistic infections [2]. More than 90% of patients with HIV have been infected at some time with cytomegalovirus. It is therefore important to identify HIV-infected patients with low CD4 cell counts who are at greatest risk of developing cytomegalovirus disease and to treat HIV effectively before cytomegalovirus disease becomes established.

Little is known about the role of virus-specific CD4T cells in maintaining immunity against cytomegalovirus [3]. It is now possible to quantitate antigen-specific CD4 lymphocytes by flow cytometry [4]. Using this method, we studied cytomegalovirus-specific CD4 lymphocyte responses in HIV-negative and HIV-infected individuals after in-vitro stimulation with lysate of cytomegalovirus-infected human fibroblasts. For each analysis, cells were gated on CD4 cell expression and up to 20 000 gated events were acquired for the detection of responding versus non-responding T cells after 6 h of in-vitro antigen stimulation. Responding CD4 T cells were defined by intracellular TNF-α or IFN-γ production and CD69 expression. Quantitation was corrected for background reactivity seen with control antigen stimulation.

In 10 healthy cytomegalovirus-positive HIV-negative individuals, cytomegalovirus-specific T cells were detected at a mean responder frequency of 1.16/μl CD69 CD4 T cells of peripheral blood IFN-γ and 1.15/μl TNF-α-producing T cells (Fig. 1, group G). The staining could be inhibited by pre-incubation with blocking monoclonal antibodies to IFN-γ and TNF-α. As expected, cytomegalovirus-specific CD4 T cells could not be demonstrated in three healthy HIV-negative cytomegalovirus-negative individuals and in 10 HIV-positive cytomegalovirus-negative subjects (Fig. 1, group F).

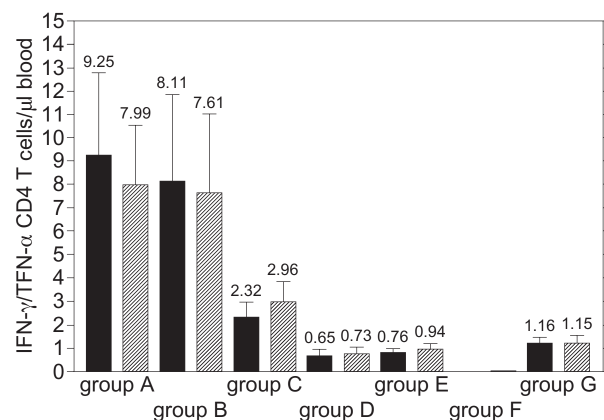

Fig. 1. Cytomegalovirus-specific CD4 T cells per microlitre of peripheral blood in HIV-infected (group A: long-term non-progressors; group B: untreated; group C: highly active antiretroviral therapy less than one year; group D: highly active antiretroviral therapy more than one year with detectable viral load; group E: highly active antiretroviral therapy more than one year without detectable viral load; group F: cytomegalovirus seronegative) and HIV-negative control group (group G). Filled bars: IFN-γ-producing CD4 CD69 T cells; hatched bars: TNF-α-producing CD4 CD69 T cells. After the subtraction of background reactivity seen with control lysate, the percentage of responding cells was multiplied with the absolute CD4 cell number, resulting in the absolute number of antigen-specific cells. There are no significant differences between IFN-γ and TNF-α-producing CD4 T-cell numbers within the same group. Mean numbers are indicated, standard error of the mean is shown above the bars.

However, the frequency of cytomegalovirus-specific CD4 T cells producing IFN-γ and TNF-α from 45 HIV-positive cytomegalovirus-positive individuals was significantly increased ($P = 0.009$; Mann–Whitney) compared with the HIV-negative population.

For a more detailed analysis, HIV-positive cytomegalovirus-positive subjects were divided into five groups according to time since initiation of antiretroviral therapy and HIV viral load: None of the patients had detectable cytomegalovirus viraemia in the Roche Amplicor assay or clinical signs suspicious of cytomegalovirus disease.

Group A consisted of three HIV-infected long-term non-progressors defined as asymptomatic, untreated HIV-1 infection for more than 10 years with CD4 cell counts greater than 500 cells/μl (mean 655/μl) and viral loads of less than 10 000 copies/ml (mean 188). Cytomegalovirus-specific T cells were detected in all HIV-positive individuals at a mean frequency of 9.25/μl IFN-γ and 7.99/μl TNF-α-producing T cells (Fig. 1, group A).

Group B consisted of nine HIV-infected subjects without antiretroviral treatment. (CD4 T-cell counts 88–1502/μl, mean 558/μl; viral loads 114–367 000 copies/ml, mean 77 289 copies/ml). Mean cytomegalovirus-specific CD4 T cells in this group were 8.11/μl blood IFN-γ-producing CD4 T cells and 7.61/μl blood TNF-α-producing CD4 T cells (Fig. 1, group B).

Group C consisted of 10 HIV-infected individuals on antiretroviral therapy for less than one year (CD4 T-cell counts 165–629/μl, mean 348/μl; viral loads < 50–1080 copies/ml, mean 226 copies/ml). In this group the mean responder frequencies were 2.32/μl IFN-γ and 2.96/μl TNF-α.

HIV-1-infected subjects treated with highly active antiretroviral therapy (HAART) for more than one year were divided into two groups: group D with detectable viral loads (CD4 T-cell counts 75–615/μl, mean 335/μl; viral loads 104–75 000 copies/ml, mean 17 726 copies/ml) and group E with undetectable viral loads (CD4 T-cell counts 117–911/μl, mean 427/μl; viral loads < 50 copies/ml). The mean cytomegalovirus-specific CD4 T-cell frequency was 0.65/μl blood for IFN-γ-producing CD4 T cells and 0.73/μl blood for TNF-α-producing CD4 T cells in group D. In group E, we detected 0.76/μl blood IFN-γ-producing CD4 T cells and 0.94/μl blood TNF-α-producing CD4 T cells. There were no significant ($P > 0.05$, Mann–Whitney) differences in cytomegalovirus responses in long-term treated patients between group D and group E.

We expected to find reduced cytomegalovirus-specific CD4 T cells in HIV patients compared with HIV-uninfected subjects. Surprisingly, the number of cytomegalovirus-specific CD4 T cells from HIV-positive cytomegalovirus-positive individuals was significantly increased, and the frequency distribution within these groups of HIV-positive individuals was broad (range 0–11.29%, mean 0.85%) compared with the HIV-negative population. Among the 45 HIV-positive cytomegalovirus-positive individuals tested, 11 (24.4%) had a more than threefold increase in the frequency of IFN-γ-producing cytomegalovirus-specific T cells over the mean frequency observed in HIV-negative subjects for IFN-γ. Only 13 (28.8%) of the remaining HIV-positive cytomegalovirus-positive subjects revealed lower numbers than the lowest observed in HIV-negative subjects.

We found strong cytomegalovirus-specific CD4 T-cell responses in untreated HIV disease. This observation may reflect an immune response against a chronic pathogen boostered by periodic low-grade cytomegalovirus replication in latently infected hosts, in which the proliferation of cytomegalovirus-specific CD4 T cells is able to counterbalance the destruction of cytomegalovirus-activated lymphocytes caused by HIV-1, thereby maintaining an effective anti-cytomegalovirus-response as long as possible. An implication of these findings is that antigen availibility is the most important factor for the survival and function of antigen-specific T helper cells.

Cytomegalovirus-specific CD4 T cells were either undetectable or detectable at very low frequency in subjects with long-term HAART-mediated viral suppression (groups D, E). This observation supports the assumption that cytomegalovirus-specific T cells are progressively lost from the recirculating pool with prolonged HIV suppression. Cytomegalovirus-specific CD4 T cells decrease after initiating HAART as a result of the successful restoration of an effective immune response against cytomegalovirus. The subsequent reduction of cytomegalovirus antigen results in the elimination of proliferation stimuli for cytomegalovirus-specific T helper cells and the predominance of multiple other T-cell specificities [3,5].

Our results show that cytomegalovirus-specific CD4 T cells can persist with or without therapy against HIV-1 infection. This indicates that these cells can be generated during HIV-1 infection. Cytomegalovirus-specific CD4 T cells are not necessarily eliminated or suppressed by ongoing HIV replication, even when it is accompanied by a CD4 T-cell decline.

Reconstitution of effective CD4 T-cell responses against cytomegalovirus in HIV patients after HAART results in immunological control of cytomegalovirus replication, as HAART can not directly inhibit cyto-

megalovirus. These promising findings support the idea of vaccination strategies against several pathogens, and ultimately HIV itself.

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Efficacy and safety of abacavir plus efavirenz as a salvage regimen in HIV-infected individuals after 48 weeks

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The efficacy and saftey of abacavir and efavirenz plus background therapy were evaluated in 50 heavily antiretroviral pre-treated patients. In this retrospective analysis, the overall antiretroviral efficacy of abacavir and efavirenz plus background therapy was limited in the salvage situation, but immunological benefit was still achieved for most patients. The tolerability of the salvage regimen seems to be good, although the drop-out rate during the first 4 weeks of treatment was high.

Once highly active antiretroviral therapy (HAART) fails to suppress HIV RNA to undetectable levels and resistance emerges, it is difficult to find alternative combinations, as the potential for cross-resistance within all available antiretroviral drug classes is high. Therefore, the introduction of new drugs is recommended by international treatment guidelines [1]. Two new antiretroviral drugs, abacavir (a nucleoside reverse transcriptase inhibitor; NRTI) and efavirenz (a non-nucleoside reverse transcriptase inhibitor; NNRTI), became available in Germany in 1998. These two potent drugs offered the basis for a new salvage regimen for multiply pretreated patients.

For this retrospective analysis, we identified 50 patients with a long antiretroviral pre-treatment history (6.3 ± 1.8 years), who experienced virological failure on HAART and were switched to a combination of abacavir plus efavirenz and NRTI and/or a protease inhibitor (PI). All patients had received at least three different previous NRTI and two PI, 20 had also received NNRTI before (delavirdine, n = 4; nevirapine, n = 16), but all were naive to efavirenz and abacavir. NRTI and/or PI as background therapy had to be different from the drugs the patients had received before, and were chosen on an individual basis (27 single NRTI; seven double NRTI; three single PI; four double PI; six NRTI plus PI; three didanosine plus hydroxyurea). All patients were followed for 48 weeks. Besides the determination of CD4 cell counts and HIV-RNA levels, genotypic resistance testing was performed before switching in all patients who had a plasma sample available and continued therapy for more than 4 weeks (n = 35). After 24 and 48 weeks genotypic analysis was repeated in all patients failing the salvage regimen who had at least one baseline and one follow-up sample available. All treatment discontinuations and adverse events were recorded. Statistical analysis followed an intent-to-treat approach. Comparison with baseline values was performed using the *t*-test for paired data. Differences between different treatment groups were tested using Fisher's exact test. Multiple logistic regression was used to explore the effects of various baseline characteristics on the probability of virological response.

The baseline CD4 cell count was 229 ± 180/μl (mean ± SD), viral load 4.75 ± 0.83 log copies/ml. Nineteen of the study subjects discontinued treatment during the 48 week follow-up (38%). Nine of these discontinuations were considered to be related to study drugs, which all occured within the first 4 weeks of treatment (rash caused by efavirenz, n = 5; abacavir hypersensitivity, n = 2; rash of unknown aetiology, n = 1; efavirenz neurotoxicity, n = 1). Four treatment discontinuations were caused by virological failure and six had other causes, which were in detail: progression of HIV infection (sepsis); renal insufficiency; stop for the treatment of tuberculosis; didanosine-associated pancreatitis; one patient stopped for his own reasons.

CD4 cell counts increased significantly by 86 cells/μl after 48 weeks ($P < 0.05$). The viral load decreased by 1.6 log copies/ml ($P < 0.05$) after the same period. A total of 19% of all patients were below the limit of

detection after 48 weeks in the observed data analysis, whereas only 12% were below 80 copies/ml after an intention-to-treat approach.

Genotypic analysis revealed that patients who responded to therapy at week 48 (< 80 copies/ml) had fewer total mutations (median three versus six; $P = 0.02$) and fewer NRTI mutations (median one versus four; $P = 0.03$) and no NNRTI mutation at baseline. All but one patient with previous NNRTI experience had NNRTI-specific mutations at baseline.

The evaluation of those patients who subsequently failed on the salvage regimen showed that 12 patients developed new NNRTI mutations (L100I, n = 2; K103N, n = 10; V106A, n = 3; V108I, n = 1; Y188C, n = 2; G190A, n = 1), whereas three patients did not develop new NNRTI mutations. However, these three patients did have significant pre-existing NNRTI mutations. No relevant changes in NRTI or PI mutations could be found.

The results of multivariate analysis to identify possible baseline characteristics for the probability of a complete viral suppression at 48 weeks demonstrated that only the number of total baseline mutations independently and significantly increased the odds of a complete virological success ($P < 0.01$; odds ratio 2.8; 95% confidence interval 1.6–6.6). Notably, previous NNRTI treatment did not reach statistical significance. Other factors not predictive of virological success were sex; baseline CD4 cell count; baseline viral load; NNRTI experience; concurrent PI use; concurrent NRTI use (one versus more than one); number of baseline NRTI mutations; number of baseline PI mutations (two or less versus more than two) and number of baseline NNRTI mutations (one or less versus more than one).

Blood glucose and cholesterol levels were within the normal range at baseline and did not change throughout the observation period at 48 weeks. Triglyceride levels were elevated at the beginning and were not affected significantly by the new salvage regimen. There was no difference between patients receiving or not a PI in their background treatment.

This retrospective analysis of heavily pretreated HIV patients demonstrates that the rate of virological failure was high, although two new, very potent drugs were administered. The observed modest antiretroviral efficacy of the abacavir plus efavirenz salvage regimen is lower than in most previous studies [2,3], in which response rates of 38–58% have been reported after shorter observation periods (24 weeks). However, similar low response rates were described (one out of 23 patients) by Khanna *et al.* [4]. Nevertheless, in all studies the majority of patients showed a significant increase in CD4 cell counts, possibly reflecting an immunological benefit despite ongoing viral replication.

Although the drop-out rate caused by skin rashes/hypersensitivity is high during the first 4 weeks of treatment, long-term tolerability appears to be good. In our experience, tolerability might be even better, as after 2 years of experience with both drugs many patients would continue treatment despite the development of a rash.

Despite some limitations (e.g. no standardized or randomized choice of the background regimen, no assessment of adherence), these data indicate that salvage therapy remains one of the critical issues in the treatment of HIV infection, and that further new drug classes as well as drugs with different resistance patterns are urgently needed.

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The effect of AIDS on maternal mortality in Malawi and Zimbabwe

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The AIDS epidemic in sub-Saharan Africa has had a significant impact on levels of pregnancy-related mortality. Nationally representative surveys in Malawi and Zimbabwe indicated that pregnancy-related mortality risks have increased 1.9 and 2.5 times, respectively, during the past decade, concomitant with a nearly 10-fold increase in the prevalence of HIV among pregnant women. The increase is more pronounced in

urban populations. These observations have important implications for intervention strategies and monitoring in the context of safe motherhood programmes.

In many countries in sub-Saharan Africa high levels of maternal mortality now co-exist with high levels of HIV prevalence among women of childbearing ages [1]. HIV/AIDS may increase pregnancy-related mortality rates through an increase in direct (e.g. puerperal infection) or indirect obstetric deaths (e.g. anemia or tuberculosis aggravated by pregnancy, or HIV progression itself being worsened by pregnancy), but HIV may also be incidental to the pregnancy (i.e. fortuitous death). Little empirical evidence exists on the interaction between HIV/AIDS and pregnancy. In a population-based longitudinal study in Rakai, Uganda [2], maternal mortality was 1687 and 310 per 100 000 live births among HIV-positive and HIV-negative women, respectively. In hospitals in South Africa, indirect causes accounted for as many as half of all maternal deaths, and AIDS was the second most common cause of maternal death [3]. Maternal mortality among HIV-infected women in a tertiary hospital in Durban, South Africa, was more than double the mortality rate among HIV-negative women, and the attributable fraction of overall deaths as a result of HIV infection was 15.9%. Mortality risks were most elevated among women with HIV–tuberculosis co-infection [4]. In a university teaching hospital in Zambia, maternal mortality rates increased considerably during the 1990s, and the increase was mainly caused by non-obstetric causes, notably malaria, HIV-associated tuberculosis, and unspecified chronic respiratory illnesses [5].

During the 1990s, Malawi and Zimbabwe each conducted two nationally representative demographic and health surveys that permit the measurement of pregnancy-related mortality. Women aged 15–49 years were asked to provide a detailed account of the survivorship of all the live-born children of their mother (maternal sibling history), including questions on whether or not the death of each deceased sister occurred during pregnancy, childbirth or the puerperium. These data allow a direct estimate of the levels of all-cause adult and pregnancy-related mortality for the period 5–10 years preceding the survey [6]. A pregnancy-related death is the death of a woman when pregnant or within 6 weeks of the termination of pregnancy, irrespective of the cause of death. The maternal mortality ratio (i.e. pregnancy-related deaths per 100 000 live births) is the pregnancy-related mortality rate divided by the general fertility rate.

In Malawi, the pregnancy-related mortality and overall adult female mortality rates were much higher in the later than in the earlier period (rate ratios 1.72 and 1.76, respectively) (Table 1). As fertility declined marginally, the maternal mortality ratio increased 1.81 times. In Zimbabwe, the large increase in overall female mortality exceeded the increase in pregnancy-related mortality rates (rate ratios 2.80 and 2.12, respectively). The maternal mortality ratio increased 2.5 times.

The increase in pregnancy-related mortality is large and significant, and erases any potential gain in maternal survival achieved by safe motherhood programmes during the preceding decade. What is the cause of the increased risks of pregnancy-related death during the 1990s in Malawi and Zimbabwe?The simplest explanation would be that the increase in pregnancy-related mortality is caused by an increase in HIV prevalence among pregnant and parturient women. Estimates of HIV prevalence, based on data from the national antenatal clinic-based surveillance system, indicate that HIV prevalence among pregnant women in Malawi was approximately 2% during 1986–1992, and approximately 15% during 1994–2000. Assuming that pregnancy-related mortality among HIV-negative women has remained constant over time, and all the increase is caused by HIV, then maternal mortality

Table 1. Period estimates of adult female mortality, maternal mortality rate, general fertility rate and maternal mortality ratio in Malawi and Zimbabwe.

Country	Year of survey	Number of respondents (women 15–49 years)	Period of estimates	Overall mortality per 1000 women 15–49 years (person years)	Pregnancy-related mortality per 1000 women 15–49 years (person years)	General fertility rate per 1000 women 15–49 years	Maternal mortality per 100 000 live births (95% CI)[a]
Malawi	1992	4849	1986–1992	6.5 (51 951)	1.4 (51 951)	0.220	620 (410–830)
	2000	13 220	1994–2000	11.3 (145 174)	2.4 (145 174)	0.210	1120 (950–1288)
			Rate ratio[b]	1.76 (1.56–1.99)	1.72 (1.33–2.25)		1.81 (1.39–2.37)
Zimbabwe	1994	6128	1985–1994	3.3 (114 169)	0.5 (114 169)	0.162	283 (195–371)
	1999	5907	1995–1999	9.1 (58 052)	0.9 (58 052)	0.135	695 (471–919)
			Rate ratio[b]	2.80 (2.45–3.20)	2.12 (1.42–3.17)		2.50 (1.68–3.72)

CI, Confidence interval.
[a]Maternal mortality reported in demographic and health surveys is standardized on the household age structure.
[b]Age-adjusted rate ratio with 95% confidence limits.

would have been 4389 per 100 000 live births among HIV-infected women (corresponding to a relative risk of 8.1 compared with HIV-negative women). The corresponding figures for Zimbabwe, with an estimated increase of HIV prevalence among pregnant women from 3% during 1983–1992 to 25% during 1995–1999, are a maternal mortality ratio of 2100 per 100 000 live births to HIV-positive women and a relative risk of 9.3.

One might expect a smaller increase in women's mortality risk by HIV status during the maternal period than outside the maternal period. In several studies, the risk of mortality among HIV-infected women during the year postpartum is considerably lower than during other periods of adulthood [7]. Selection factors are probably the primary explanation as pregnant women may be healthier [8] and at less advanced stages of HIV infection [9]. In Malawi, the increase in pregnancy-related mortality is proportional to the increase in all-cause mortality, suggesting that pregnancy and delivery may carry additional mortality risks that offset the advantages generated by the selection bias. In Zimbabwe, where the AIDS epidemic is even more severe than in Malawi, the mortality increase was larger among all causes than among pregnancy-related causes, which could be partly due to the effect of HIV on fertility, as a substantial decrease in fertility was observed in Zimbabwe (Table 1). The decrease in fertility reduces the maternal mortality rate, but has little effect on the ratio.

Surveillance data from antenatal clinics indicate that urban populations were affected earlier and more severely than rural populations during the 1990s. Therefore, one expects a larger increase in maternal mortality rates in urban areas, which is indeed the case in both countries (Fig. 1). Note that no data were collected on the residence of the siblings. Therefore, the maternal mortality data in Fig. 1 are by the residence of the respondents and not by the residence of the sisters, which would be more accurate.

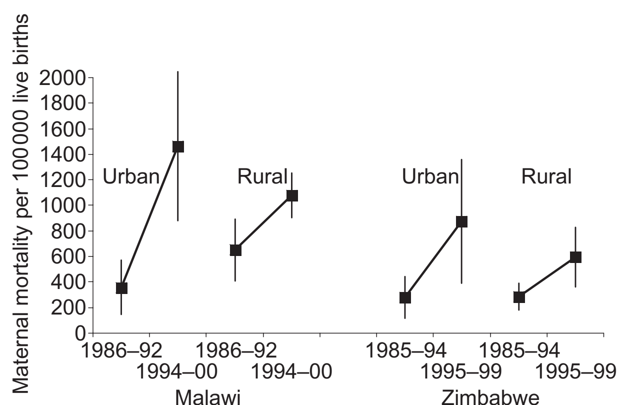

Fig. 1. Maternal mortality ratio trends in Malawi and Zimbabwe during the 1990s (period estimate with 95% confidence limits).

Two additional explanations need to be considered. There may be a tendency to report the deaths of women of reproductive ages as maternal deaths to avoid the stigmatization associated with a possible AIDS death. Another explanation is that obstetric risk has indeed worsened because of a deterioration in the quality of delivery services. Even if safe motherhood programmes increase the awareness of complications among women and provide information and services to anticipate and respond to problems, HIV-related illnesses may increase crowding in health facilities and affect the quality of maternity services. The demographic and health surveys in both countries do not indicate changes in the utilization of antenatal or delivery care. For instance, the proportion of pregnant women delivering in health facilities remained approximately 55 and 72% in Malawi and Zimbabwe, respectively.

Clearly, further study is required to assess how HIV/AIDS affects pregnancy-related mortality rates. To distinguish the effects of the HIV epidemic from the progress in access to and quality of obstetric services, maternal mortality measurements should separate direct obstetric from other causes. Furthermore, it is essential to consider the probable influence of HIV on maternal outcomes, health service delivery and the behaviour of women. Collaborative initiatives to reduce HIV and maternal mortality need to be developed, and programmes that aim to reduce mother-to-child transmission of HIV are the most logical entry point.

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